TITLE

NOVEL NUCLEOTIDE SEQUENCES ENCODING ACC SYNTHASE ENZYMES"

This application is a continuation of U.S. Application Serial No.09/043,627 filed March 20, 1998, now U.S. Patent No. 6,124,525, which was a 35 USC §371 application of PCT/AU96/00591, filed September 20, 1996 and amended under PCT Article 34 on June 10, 1997.

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FIELD OF THE INVENTION

This invention relates to ACC synthase and, in particular, novel nucleotide sequences encoding ACC synthase enzymes derived from pineapple, papaya and mango.

BACKGROUND OF THE INVENTION

Ethylene is a well-established plant hormone. It plays an important role in virtually every phase of plant development including seed germination, fruit ripening, leaf and flower senescence, and abscission. The production of ethylene may also be induced by external factors such as mechanical wounding, anaerobiosis, auxin treatment, ultraviolet light, temperature extremes, water stress, and ions such as cadmium, and lithium ions (Abeles, F.B., 1973, Ethylene in Plant Biology, 197-219, Academic Press, London; Yang & Hoffman, 1984, Annu. Rev. Plant Physiol., **35**, 155-189).

The pathway for ethylene biosynthesis has been established, the first step of which involves the formation of *S*-adenosyl-L-methionine (AdoMet) by *S*-adenosyl-L-methionine synthetase. AdoMet is subsequently converted by *S*-adenosyl-L-methionine methylthio-adenosine-lyase (ACC synthase; EC 4.4.1.14) to the nonprotein amino acid 1-aminocyclopropane-1 carboxylic acid (ACC), the immediate precursor of ethylene in higher plants (Adams & Yang, 1979, Proc. Natl. Acad. Sci. USA, **76**, 170-174). Physiological analysis has suggested that this is the key regulatory step in the pathway, (Kende, 1989, Plant Physiol., **91**, 1-4). Thus, the rate of endogenous expression of ACC synthase is considered to limit substantially the rate of ethylene production.

It appears that ACC synthase is encoded by a highly divergent multigene family (for a review, see Theologis, A. 1992, Cell, **70**, 181-184; Kende, H., 1993, Annu. Rev. Plant Physiol. Plant Mol. Biol., **44**, 283-307). In tomato, for example, ACC

synthase is encoded by at least six genes, two of which are expressed in fruit ripening (Van der Straeten *et al.*, 1990, Proc. Natl. Acad. Sci. USA, **87**, 4859-4863; Olson *et al.*, 1991, Proc. Natl. Acad. Sci. USA, **88**, 5340-5344; Rottmann *et al.*, 1991, J. Mol. Biol., **222**, 937-961; Yipp *et al.*, 1992, Proc. Natl. Acad. Sci. USA, **89**, 2475-2479).

In addition, reference may be made to an article by Theologis, A. (1992, *supra*) in which the structure and expression of 20 ACC synthase genes were compared from a variety of plant species including winter squash, zucchini, tomato, Arabidopsis, apple, rice, mung bean and carnation. This comparison suggested that the extensive polymorphism and distinct regulatory networks governing the expression of ACC synthase subfamilies arose early in plant evolution, prior to the divergence of monocotyledons and dicotyledons.

It is well known that endogenous ethylene is often deleterious to crops. In particular, increased ethylene production due to trauma caused by mechanical wounding of fruits and vegetables, and the cutting of flowers greatly diminishes their post harvest quality and storage life. Thus, it has been a major goal of postharvest physiologists to effect suppression of fruit ripening and flower fading by inhibiting the biosynthesis of ethylene.

To this end, a strategy has been developed that takes advantage of the modulation properties of ACC synthase in the control of ethylene biosynthesis. In this regard, reference may be made to International Application Publication No. WO 92/04456 which is directed to inhibition of expression of endogenous ACC synthase using an antisense expression system. This system comprises a DNA molecule capable of generating, when contained in a plant host cell, a complementary RNA that is sufficiently complementary to an RNA transcribed from an endogenous ACC synthase gene to prevent the synthesis of endogenous ACC synthase. Ethylene production in fruits of transgenic tomato plants engineered using this system was inhibited by 99.5% and, as a consequence, fruit ripening was suppressed. In addition, the application of ethylene or propylene to the fruits of these plants restored normal ripening.

Thus, ACC synthase genes may be used as targets for the generation of transgenic plants in which endogenous expression of ACC synthase is inhibited to effect suppression of ethylene production and a concomitant delay in fruit ripening.

The efficacy of this system, however, is predicated on the condition that

the antisense RNA is sufficiently complementary to the transcript expressed from the target gene. Accordingly, if there is diversity between different ACC synthase genes, the use in this system for example, of a particular ACC synthase gene from one plant species would not be expected to inhibit the expression of an ACC synthase gene from another plant species. This is supported on page 5 of WO 92/04456 which states: "While the various ACC synthases are generally active in a variety of plant tissues, the DNAs are not completely homologous, and therefore the use of the genetic materials for control of synthesis, for example, using an antisense strategy, does not translate cross species."

SUMMARY OF THE INVENTION

It is therefore an object of the invention to provide new nucleotide sequences encoding ACC synthase enzymes which have utility as targets for the generation of transgenic plants in which the expression of ACC synthase is substantially controlled to effect the regulation of plant development, and in particular, fruit ripening.

Accordingly, in one aspect of the invention, there is provided a nucleotide sequence encoding an ACC synthase enzyme of pineapple comprising the sequence of nucleotides as shown in FIG. 1.

In another aspect of the invention, there is provided a nucleotide sequence encoding a first ACC synthase enzyme of papaya comprising the sequence of nucleotides as shown in FIG. 2.

In yet another aspect of the invention, there is provided a nucleotide sequence encoding a second ACC synthase enzyme of papaya comprising the sequence of nucleotides as shown in FIG. 3.

In still yet another aspect of the invention, there is provided a nucleotide sequence encoding a first ACC synthase enzyme of mango comprising the sequence of nucleotides as shown in FIG. 4.

In a further aspect of the invention, there is provided a nucleotide sequence encoding a second ACC synthase enzyme of mango comprising the sequence of nucleotides as shown in FIG. 5.

The term "nucleotide sequence" as used herein designates mRNA, RNA, cRNA, cDNA or DNA.

The invention also provides homologs of the nucleotide sequences of the

invention described in FIGS. 1-5. Such "homologs", as used in this specification include all nucleotide sequences encoding sub-sequences of the nucleotide sequences according to FIGS 1-5.

The homologs of the invention further comprise nucleotide sequences that hybridize with any one of the nucleotide sequences of the invention under stringent conditions. Suitable hybridization conditions are discussed below.

The homologs of the invention may be prepared according to the following procedure:

(i) designing primers which are preferably degenerate which span at least a fragment of a nucleotide sequence of the invention; and

(ii) using such primers to amplify, via PCR techniques, said at least a fragment from a nucleic acid extract obtained from a suitable host. In this regard, the suitable host is preferably a fruit, fruit part or cell thereof obtained from a pineapple plant, a mango plant or a papaya plant.

BRIEF DESCRIPTION OF THE DRAWINGS

In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of example with reference to the following figures in which:

FIG. 1 (SEQ ID NOS:1 and 2 respectively) shows the nucleotide and deduced amino acid sequences encoding an ACC synthase enzyme of pineapple;

FIG. 2 (SEQ ID NOS:3 and 4 respectively) shows the nucleotide and deduced amino acid sequences encoding a first ACC synthase enzyme of papaya;

FIG. 3 (SEQ ID NOS:5 and 6 respectively) shows the nucleotide and deduced amino acid sequences encoding a second ACC synthase enzyme of papaya;

FIG. 4 (SEQ ID NOS:7 and 8 respectively) shows the nucleotide and deduced amino acid sequences encoding a first ACC synthase enzyme of mango; and

FIG. 5 (SEQ ID NOS:9 and 10 respectively) shows the nucleotide and deduced amino acid sequences encoding a second ACC synthase enzyme of mango.

DETAILED DESCRIPTION OF THE INVENTION

"Hybridization" is used here to denote the pairing of complementary nucleotide

sequences to produce a DNA-DNA hybrid or a DNA-RNA hybrid. Complementary base sequences are those sequences that are related by the base-pairing rules. In DNA, A pairs with T and C pairs with G. In RNA, U pairs with A and C pairs with G.

Typically, nucleotide sequences to be compared by means of hybridization are analyzed using dot blotting, slot blotting, or Southern blotting. Southern blotting is used to determine the complementarity of DNA sequences. Northern blotting determines complementarity of DNA and RNA sequences. Dot and Slot blotting can be used to analyze DNA/DNA or DNA/RNA complementarity. These techniques are well known by those of skill in the art. Typical procedures are described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Ausubel, *et al.*, eds.) (John Wiley & Sons, Inc. 1995) at pages 2.9.1 through 2.9.20. Briefly, for Southern blotting, DNA samples are separated by size using gel electrophoresis. The size-separated DNA samples are transferred to and immobilized on a membrane (typically, nitrocellulose) and the DNA samples are probed with a radioactive, complementary nucleic acid. In dot blotting, DNA samples are directly spotted onto a membrane (nitrocellulose or nylon). In slot blotting, the spotted DNA samples are elongated. The membrane is then probed with a radioactive complementary nucleic acid.

A probe is a biochemical labeled with a radioactive isotope or tagged in other ways for ease in identification. A probe is used to identify a gene, a gene product or a protein. Thus a nucleotide sequence probe can be used to identify complementary nucleotide sequences. An mRNA probe will hybridize with its corresponding DNA gene.

Typically, the following general procedure can be used to determine hybridization under stringent conditions. A nucleotide sequence according to the invention (such as those shown in FIGS. 1-5 or a sub-sequence thereof) will be immobilized on a membrane using one of the above-described procedures for blotting. A sample nucleotide sequence will be labeled and used as a "probe." Using procedures well known to those skilled in the art for blotting described above, the ability of the probe to hybridize with a nucleotide sequence according to the invention can be analyzed.

One of skill in the art will recognize that various factors can influence the amount and detectability of the probe bound to the immobilized DNA. The specific activity of the probe must be sufficiently high to permit detection. Typically, a specific activity of at least 10^8 dpm/μg is necessary to avoid weak or undetectable hybridization

signals when using a radioactive hybridization probe. A probe with a specific activity of 10^8 to 10^9 dpm/μg can detect approximately 0.5 pg of DNA. It is well known in the art that sufficient DNA must be immobilized on the membrane to permit detection. It is desirable to have excess immobilized DNA and spotting 10 μg of DNA is generally an acceptable amount that will permit optimum detection in most circumstances. Adding an inert polymer such as 10% (w/v) dextran sulfate (mol. wt. 500,000) or PEG 6000 to the hybridization solution can also increase the sensitivity of the hybridization. Adding these polymers has been known to increase the hybridization signal. See Ausubel, *supra*, at p 2.10.10.

To achieve meaningful results from hybridization between a first nucleotide sequence immobilized on a membrane and a second nucleotide sequence to be used as a hybridization probe, (1) sufficient probe must bind to the immobilized DNA to produce a detectable signal (sensitivity) and (2) following the washing procedure, the probe must be attached only to those immobilized sequences with the desired degree of complementarity to the probe sequence (specificity).

"Stringency," as used in this specification, means the condition with regard to temperature, ionic strength and the presence of certain organic solvents, under which nucleic acid hybridizations are carried out. The higher the stringency used, the higher degree of complementarity between the probe and the immobilized DNA.

"Stringent conditions" designates those conditions under which only nucleotide sequences that have a high frequency of complementary base sequences will hybridize with each other.

Exemplary stringent conditions are (1) 0.75 M dibasic sodium phosphate/0.5 M monobasic sodium phosphate/1 mM disodium EDTA/1% sarkosyl at about 42°C for at least about 30 minutes, (2) 6.0 M urea/0.4% sodium lauryl sulfate/0.1% SSC at about 42° C for at least about 30 minutes, (3) 0.1 X SSC/0.1% SDS at about 68°C for at least about 20 minutes, (4) 1 X SSC/0.1% SDS at about 55°C for about one hour, (5) 1 X SSC/0.1% SDS at about 62°C for about one hour, (6) 1 X SSC/0.1% SDS at about 68°C for about one hour, (7) 0.2 X SSC/0.1% SDS at about 55°C for about one hour, (8) 0.2 X SSC/0.1% SDS at about 62°C for about one hour, and (9) 0.2 X SSC/0.1% SDS at about 68°C for about one hour. *See, e.g.* CURRENT

PROTOCOLS IN MOLECULAR BIOLOGY (Ausubel, *et al.*, eds.) (John Wiley & Sons, Inc. 1995), pages 2.10.1-2.10.16 of which are hereby incorporated by reference and Sambrook, *et al.*, MOLECULAR CLONING. A LABORATORY MANUAL (Cold Spring Harbor Press, 1989) at §§1.101-1.104.

Stringent washes are typically carried out for a total of about 20 minutes to about 60 minutes. In certain instances, more than one stringent wash will be required to remove sequences that are not highly similar to the nucleotide sequences shown in FIGS. 1-5 or a sub-sequence thereof. Typically, two washes of equal duration, such as two 15 or 30 minute washes, are used. One of skill in the art will appreciate that other longer or shorter times may be employed for stringent washes to ensure identification of sequences similar to the nucleotide sequences designated in FIGS. 1-5.

While stringent washes are typically carried out at temperatures from about 42°C to about 68°C, one of skill in the art will appreciate that other temperatures may be suitable for stringent conditions. Maximum hybridization typically occurs at about 20 to about 25°C below the T_m for DNA-DNA hybrids. It is well known in the art that T_m is the melting temperature, or temperature at which two nucleotide sequences dissociate. Methods for estimating T_m are well known in the art. *See, e.g.* Ausubel, *supra*, at page 2.10.8. Maximum hybridization typically occurs at about 10 to about 15°C below the T_m for DNA-RNA hybrids.

Other typical stringent conditions are well-known in the art. One of skill in the art will recognize that various factors can be manipulated to optimize the specificity of the hybridization. Optimization of the stringency of the final washes can serve to ensure a high degree of hybridization between the nucleotide sequences shown in FIGS. 1-5 (or sub-sequence thereof) and other similar nucleotide sequences.

In a typical hybridization procedure, DNA is first immobilized on a membrane such as a nitrocellulose membrane or a nylon membrane. Procedures for DNA immobilization on such membranes are well known in the art. See, *e.g.*, Ausubel, *supra* at pages 2.9.1-2.9.20. The membrane is prehybridized at 42°C for 30-60 minutes in 0.75 M dibasic sodium phosphate/0.5 M monobasic sodium phosphate/1 mM disodium EDTA/1% sarkosyl. Membranes are then hybridized at 42°C in ACES hybridization solution (Life Technologies, Inc., Gaithersburg, Md.) containing labeled

probe for one hour. Next, membranes are subjected to two high stringency 10 minute washes at 42°C in 0.75 M dibasic sodium phosphate/0.5 M monobasic sodium phosphate/1 mM disodium EDTA/1% sarkosyl. Following this, the membranes are washed with 2 X SSC at room temperature, to remove unbound probe.

In another typical hybridization procedure, DNA immobilized on a membrane is hybridized overnight at 42°C in prehybridization solution. Following hybridization, blots are washed with two stringent washes, such as 6.0 M urea/0.4% sodium lauryl sulfate/0.1% SSC at 42°C. Following this, the membranes are washed with 2 X SSC at room temperature.

Autoradiographic techniques for detecting radioactively labeled probes bound to membranes are well known in the art.

This invention further includes within its scope homologs which comprise synonymous DNA sequences that code for the ACC synthase enzymes or portions thereof shown in FIGS. 1-5.

In another aspect, the invention resides in a method of isolating at least a fragment of an ACC synthase gene involved in regulation of fruit ripening, said method including the steps of:-

(a) lysing ripened tissue of a fruit to produce a lysate;

(b) separating protein and carbohydrates from the lysate to produce an extract comprising substantially intact RNA wherein said extract is substantially incapable of inhibiting cDNA synthesis;

(c) reverse transcribing the RNA of said extract with an antisense primer complementary to a portion of said ACC synthase gene to synthesize a cDNA; and

(d) subjecting the cDNA to PCR with a sense primer and an antisense primer which are complementary to different portions of said ACC synthase gene to amplify said at least a fragment of said ACC synthase gene.

Preferably, the fruit is pineapple, papaya or mango.

Suitably, the step of lysing (step (a)) is effected by lysing the ripened tissue in a medium comprising 150 mM Tris pH 7.5 with boric acid, 2% SDS, 50 mM EDTA, 1% mercaptoethanol. Preferably, 2-3 volumes of this medium is used per volume of ripened tissue.

Preferably, the step of separating (step (b)) is effected by at least one chloroform-isoamyl alcohol extraction of the lysate followed by at least two phenol-chloroform extractions of an aqueous phase resulting from the at least one chloroform-isoamyl alcohol extraction. Alternatively, the step of separating may be effected by at least one chloroform-isoamyl alcohol extraction of the lysate followed by at least two phenol-chloroform-isoamyl alcohol extractions of an aqueous phase resulting from the at least one chloroform-isoamyl alcohol extraction.

The primers used for reverse transcription (step (c)) and PCR (step (d)) are preferably degenerate primers. Suitably, the degenerate primers correspond to conserved portions of different ACC synthase isoforms. Preferably, the degenerate primers are selected from the group consisting of:

5' TA(C/T)TT(C/T)GA(C/T)GG(A/C/G/T)TGGAA(A/G)GC 3' (SEQ ID NO:11);

5' TC(A/G)TCCAT(A/G)TT(A/C/G/T)GC(A/G)AA(A/G)CA 3' (SEQ ID NO:12);

5' CA(A/G)ATGGG(A/C/G/T)(C/T)T(A/C/G/T)GC(A/C/G/T)GA(A/G)AA 3'(SEQ ID NO:13); 5' AC(A/C/G/T)C(G/T)(A/G)AACCA(A/C/G/T)CC(A/C/G/T)GG(C/T)TC 3' (SEQ ID NO:15); 5' GCTCTAGATA(C/T)TT(C/T)GA(C/T)GG(A/C/G/T)TGGAA(A/G)GC 3' (SEQ ID NO:16); 5' GCGAATTC(A/G)TCCAT(A/G)TT(A/C/G/T)GC(A/G)AA(A/G)CA 3' (SEQ ID NO:17);5' CCTGATCA(A/G)ATGGG(A/C/G/T) (C/T)T(A/C/G/T)GC(A/C/G/T)GA(A/G)AA 3' (SEQ ID NO:18); and 5' CTCTGCAGC(A/G)AA(A/G)CA(A/C/G/T)AC(A/C/G/T)C(G/T)(A/G)A CCA 3' (SEQ ID NO:19).

Preferably, when the fruit is pineapple, the antisense primer for reverse transcription is 5' TC(A/G)TCCAT(A/G)TT(A/C/G/T)GC(A/G) AA(A/G)CA 3' (SEQ ID NO:12), the sense primer for PCR is 5' TA(C/T)TT(C/T) GA(C/T)GG(A/C/G/T)TGGAA(A/G)GC 3'(SEQ ID NO:11) or 5' CA(A/G)ATGGG(A/C/G/T) (C/T)T(A/C/G/T)GC(A/C/G/T)GA(A/G)AA 3' (SEQ ID NO:13), and the antisense primer for PCR is 5' TC(A/G)TCCAT(A/G)TT(A/C/G/T)GC(A/G)AA(A/G)CA 3' (SEQ ID NO:12) or 5' AC (A/C/G/T)C(G/T)(A/G)AACCA(A/C/G/T)CC(A/C/G/T)GG(C/T)TC 3' (SEQ ID NO:15).

Suitably, when the fruit is mango or papaya, the antisense primer for reverse transcription is 5' GCGAATTC(A/G)TCCAT(A/G)TT (A/C/G/T)GC(A/G)AA(A/G)CA (SEQ ID NO:17) 3', the sense primer for PCR is 5'

GCTCTA GATA(C/T)TT(C/T)GA(C/T)GG(A/C/G/T)TGGAA(A/G)GC 3' (SEQ ID NO:16) or 5' CCTGATCA(A/G)ATGGG(A/C/G/T) (C/T)T(A/C/G/T)GC(A/C/G/T)GA(A/G)AA 3'(SEQ ID NO:18), and the antisense primer for PCR is 5' GCGAATTC(A/G) TCCAT(A/G)TT (A/C/G/T)GC(A/G)AA(A/G)CA 3' (SEQ ID NO:17) or 5' CTCTGCAGC(A/G) AA(A/G)CA(A/C/G/T)AC(A/C/G/T)C(G/T)(A/G)AACCA (SEQ ID NO:19) 3'.

Preferably, in step (d) comprises a first PCR and a second PCR wherein the second PCR employs a nested pair of primers relative to those used in the first PCR.

The DNA sequences of the invention have utility as targets for the generation of transgenic variants of pineapple, papaya and mango in which the expression of ACC synthase is substantially inhibited to effect suppression of fruit senescence.

The method of generating a transgenic pineapple with inhibited fruit senescence includes the steps of introducing into a pineapple plant, or plant part or cell thereof a vector comprising the nucleotide sequence of FIG. 1 wherein said sequence is operably linked, in the sense orientation, to one or more regulatory nucleotide sequences, and growing said plant or plant part or cell thereof to generate the transgenic variety of pineapple.

The invention also comprises a method of generating a transgenic variety of pineapple wherein fruit senescence is substantially inhibited, said method including the steps of introducing into a pineapple plant, or plant part or cell thereof a vector comprising the nucleotide sequence of FIG. 1 wherein said sequence is operably linked, in the antisense orientation, to one or more regulatory nucleotide sequences, and growing said plant or plant part or cell thereof to generate the transgenic variety of pineapple.

The method of generating a transgenic papaya plant with inhibited fruit senescence includes the steps of introducing into a papaya plant, or plant part or cell thereof a vector comprising the nucleotide sequence of FIG. 2 and/or FIG. 3 wherein said sequence is operably linked, in the sense orientation, to one or more regulatory nucleotide sequences, and growing said plant or plant part or cell thereof to generate the transgenic variety of papaya.

The invention further comprises a method of generating a transgenic variety of papaya wherein fruit senescence is substantially inhibited, said method including the steps of introducing into a papaya plant, or plant part or cell thereof a vector comprising the nucleotide sequence of FIG. 2 and/or FIG. 3 wherein said sequence is operably linked, in the antisense orientation, to one or more regulatory nucleotide sequences, and growing said plant or plant part or cell thereof to generate the transgenic variety of papaya.

The method of generating a transgenic mango plant with inhibited fruit senescence includes the steps of introducing into a mango plant, or plant part or cell thereof a vector comprising the nucleotide sequence of FIG. 4 and/or FIG. 5 wherein said sequence is operably linked, in the sense orientation, to one or more regulatory nucleotide sequences, and growing said plant or plant part or cell thereof to generate the transgenic variety of mango.

The invention still further comprises a method of generating a transgenic variety of mango wherein fruit senescence is substantially inhibited, said method including the steps of introducing into a mango plant, or plant part or cell thereof a vector comprising the nucleotide sequence of FIG. 4 and/or FIG. 5 wherein said sequence is operably linked, in the antisense orientation, to one or more regulatory nucleotide sequences, and growing said plant or plant part or cell thereof to generate the transgenic variety of mango.

A vector according to the invention may be a prokaryotic or a eukaryotic expression vector, which are well known to those of skill in the art. Such vectors may contain one or more copies of the nucleotide sequences according to the invention.

Regulatory nucleotide sequences which may be utilized to regulate expression of the nucleotide sequences of FIGS. 1-5 or homologs thereof include, but are not limited to, a promoter, an enhancer, and a transcriptional terminator. Such regulatory sequences are well known to those of skill in the art.

Suitable promoters which may be utilized to induce expression of the nucleotide sequences of the invention include constitutive promoters and inducible promoters. A particularly preferred promoter which may be used to induce such expression is the Cauliflower Mosaic Virus (CaMV) 35S promoter.

Any suitable transcriptional terminator may be used which effects

termination of transcription of a nucleotide sequence in accordance with the invention. Preferably, the nopaline synthase (NOS) terminator, as for example disclosed in United States Patent Specification No. US 5,034,322, is used as the transcription terminator.

The vector may also include a selection marker such as an antibiotic resistance gene which can be used for selection of suitable transformants. Examples of such resistance genes include the *nptII* gene which confers resistance to the antibiotics kanamycin and G418 (Geneticin®) and the *hph* gene which confers resistance to the antibiotic hygromycin B.

The vector may be introduced by a number of methods including transfection, projectile bombardment, electroporation or infection by *Agrobacterium tumefaciens*.

Of course it will be appreciated that the nucleotide sequence which is operably linked to the regulatory nucleotide sequence may be a homolog of the corresponding nucleotide sequence as described above.

With reference to the above methods wherein the nucleotide sequence is expressed in the sense orientation, such methods are predicated on RNA-mediated suppression (also termed gene-silencing or co-suppression) wherein transcription products of the nucleotide sequence which are substantially homologous to corresponding transcripts of an endogenous gene cause inhibition of expression of the endogenous gene. In this regard, reference may be made to WO 90/12084 (which is hereby incorporated by reference) which discloses methods for engineering transgenic plants based on RNA-mediated suppression.

With reference to the above methods wherein the nucleotide sequence is expressed in the antisense orientation, such methods are based on antisense-mediated suppression as, for example, described in WO 92/04456 (which is hereby incorporated by reference).

It will of course be appreciated that gene transplacement by homologous recombination may also be used to effect the generation of suitable transgenic plants. Such methods are well known to persons of skill in the art.

EXAMPLE 1

Amplification of an ACC Synthase Gene from Pineapple

Experimental Strategy:

A rapid and simple procedure for extracting efficiently intact RNA from pineapple fruit pulp, a tissue rich in polysaccharides and carbohydrates, required the modification and integration of several conventional methods (Logemann *et al.*, 1987, Anal. Biochem., **163**, 16-20; Lopez-Gomez and Gomez-Lim, 1992, HortScience, **27**, 440-442; Schultz et al., 1994, Plant Mol. Biol., Reporter **12**, 310-316; Su and Gibor, 1988, Anal. Biochem., **174**, 650-657). The method was optimised such that the RNA isolated from the plant material satisfied three criteria: (1) RNA should be substantially intact to furnish reproducible migration patterns following gel electrophoresis; (2) the yield of RNA should be sufficient so that relatively small amounts of tissue could be used; (3) the RNA should be free from any contaminants that could interfere with cDNA synthesis or Northern blots.

We developed a method fulfilling all three criteria. The aim was to extract high quality RNA from mature green and ripe pineapple fruit (*Ananas comosus* L.; cultivar; Smooth Cayenne, Queensland clone 30) flesh. The same methodology was also used to extract high molecular weight genomic DNA for southern analysis.

Materials and Methods:

All glassware, utensils and centrifuge tubes were rinsed with water containing 0.1% diethyl pyrocarbonate (DEPC) and autoclaved in order to remove RNA degrading enzymes and protein contamination. Solutions were incubated with DEPC (0.1% final concentration) overnight and autoclaved to denature any RNases present in said solutions.

Nucleic Acid Extraction Procedure for Pineapple

Pineapple fruit tissue (derived from *Ananas comosus* L.) was frozen in liquid nitrogen and stored at -70°C. In a liquid-nitrogen-filled mortar, pineapple fruit tissue (10 g) or pineapple leaf tissue (1-4 g), was ground to a fine powder. While still frozen the fruit powder was transferred to a 100 mL beaker containing 30 mL of lysis buffer (150 mM Tris pH 7.5 with boric acid, 2% SDS, 50 mM EDTA, 1% mercaptoethanol) and stirred for 5 min at room temperature. 0.25 volume of absolute ethanol and 0.11 volume of 5 M potassium acetate were added to the homogenate and

stirred for a further 3 min. One volume of chloroform isoamyl alcohol (24:1) (SEVAG) was added subsequently and mixed for an additional 3 min. The homogenate was centrifuged at 18,000 rpm for 10 min in a prechilled (4°C) centrifuge. The recovered aqueous phase was extracted twice with phenol-chloroform (1:1) or until no interphase (proteins and carbohydrates) was apparent.

After careful removal of the aqueous phase, nucleic acids were precipitated by the addition of 2.25 volumes of absolute ethanol. After incubation for 2 hrs at -20°C, nucleic acids were centrifuged for 30 min at 18,000 rpm, dried and redissolved in 10 mL of DEPC treated dH_2O. 8 M LiCl was added to a final concentration of 3 M and the mixture incubated overnight at -20°C. RNA was collected by centrifugation (18,000 rpm, for 30 min at 4°C) and washed twice in 80% ethanol at room temperature and dried. The RNA pellet was subsequently resuspended in 300 µL of DEPC treated water, and RNA precipitated by adding sodium acetate to a final concentration of 0.3 M and 2.5 volumes of absolute ethanol. After overnight incubation at -70°C, the RNA was pelleted by centrifugation for 30 min at 14,000 rpm (4°C). The pellet was washed twice in 80% ethanol and vacuum dried for 10 min. The RNA was then resuspended in 50 µL of DEPC treated sterile water, spectrophotometrically quantified and stored at -70°C.

Genomic DNA was pelleted from the supernatant collected after the LiCl precipitation step by the addition of 2 volumes of absolute ethanol, kept at -20°C for 2 hrs and centrifuged at 12,000 rpm for 30 min. The DNA pellet was resuspended in 500 µL of TE buffer, spectrophotometrically quantified and stored at -20°C.

Formaldehyde Denaturing Gel Electrophoresis of RNA

Ten µg of total RNA was electrophresed on a 1% agarose gel at 80 V for 1 hr. The gel was prepared by adding 0.5 g of agarose, 5 mL of 10x MOPS, 36 mL of DEPC treated water and 9 mL of formaldehyde. RNA samples were prepared by adding 25 µL of RNA loading buffer (containing ethidium bromide) to 5 µL of RNA and denatured at 70°C for 5 min, followed by chilling on ice for 2 min. Electrophoresis was conducted in 1x MOPS buffer. The gel was photographed immediately after electrophoresis.

DNA Gel Electrophoresis

The integrity of genomic DNA was checked by electrophoresis on a 0.8% agarose gel (prepared in 1X TBE buffer) at 80 volts for 1 hr. 10X loading buffer was added to appoximately 4 μg of DNA and electrophoresis conducted in 1X TBE buffer. The gel was stained in an ethidium bromide solution (66 μg/mL) and photographed as described above.

Design of Primers for Amplification of ACC Synthases From Higher Plants

Homology studies of several ACC synthase proteins revealed various conserved regions among this family of proteins. Those regions were used in designing degenerate oligonucleotides by reverse translating the amino acid sequence and taking in account the degeneracy of the genetic code. As a result, several degenerate oligonucleotides were synthesized and are shown in Table 1.

Amplification of acacc1

Reverse transcription of RNA from ripe pineapple tissue was performed by using 1 μg total RNA and 2.5 U of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase in a reaction mixture of 10 mM Tris-HCl, pH 8.3 containing 50 mM KCl, 5 mM $MgCl_2$, 1 mM of each dNTP, 1 U RNase inhibitor and 0.75 μM of oligonucleotide primer EZ-4 (Table 1). The reaction mixture was incubated for 30 min at 42°C and then heated to 99°C for 5 min to inactivate the reverse transcriptase. The cDNA produced was amplified with 2.5 U AmpliTaq DNA Polymerase in a reaction mixture of 10 mM Tris-HCl, pH 8.3 containing 2 mM $MgCl_2$, 50 mM KCl, and 0.15 μM of EZ-2 and EZ-4 (Table 1). After an initial 3 min denaturing period at 94°C, the PCR parameters were 1 min template denaturation at 94°C, 1 min primer annealing at 48°C and 2 min primer extension at 72°C for 45 cycles. A final extension step of 15 min at 72°C was used subsequently to ensure full length amplification products.

The products of this PCR reaction were further amplified using a second set of oligonucleotide primers EZ-5 and EZ-7 (Table 1). The reaction consisted of adding to a tube a sample of the products obtained from the previous amplification and 2.5 U AmpliTaq DNA Polymerase in a reaction mixture of 10 mM Tris-HCl, pH 8.3 containing 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dNTP, 0.1% gelatin and 0.15 μM of each primer in a total volume of 100 μL. The PCR parameters were 30 sec template

denaturation at 94°C, 30 sec primer annealing at 48°C and 1.5 min primer extension at 72°C for 30 cycles. The PCR products were analyzed on 0.8% agarose gels and visualized with ethidium bromide.

Subcloning and Sequencing of PCR Products

The cDNA amplified by PCR was ligated into pBluescript (SK^+) (Stratagene, La Jolla, CA). The ligation mixtures were used to transform *E. coli* DH5α. Transformants were selected on LB plates containing ampicillin (50 mg/ml) and X-gal (0.033% w/v). Plasmid DNA was isolated using the alkaline lysis method.

DNA sequencing was carried out using Applied Biosystems PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit.

Results:

After optimising all the parameters for the PCR amplification reactions, a DNA band of approximately 1.1 Kb could be observed in electrophoresis gels. The DNA fragment corresponding to this band was subcloned into pBluescript and named *acacc1*. The nucleotide sequence of *acacc1* and the deduced amino acid sequence thereof are shown in FIG. 1. Comparison of these sequences with other published ACC synthase sequences indicates that *acacc1* is a cDNA coding for a member of the ACC synthase family in pineapple. Searches in all the available protein and DNA data banks failed to find 100% homology with any existing clone. The highest homology found at the DNA level using the blastn program was 65% with *Nicotiana tabacum* mRNA clone # X65982 (EMBL data bank). Similarly, at the protein level the highest homology found using the program BLASTX was 65% with soybean sequence entry # S25002 in the PIR database.

Analysis of the *acacc1* DNA sequence reveals that it is 1080 bp in size and represents approximately 75% of the coding region.

EXAMPLE 2

Amplification of ACC Synthase Genes from Papaya

Materials and Methods:

Tissue preparation

Using a sharp knife, the papaya fruit (derived from *Carica papaya* L.) was

cut in half and the seeds removed. The skin was cut away and the fruit flesh was diced into small cubes which were immediately frozen in liquid nitrogen. The liquid nitrogen was drained off and the tissue was stored at -80°C.

RNA Extraction Procedure for Papaya

Three to six grams of ripe papaya fruit tissue was ground in liquid nitrogen using a pre-cooled mortar and pestle and disolved in 2 to 3 volumes of extraction buffer (150 mM Tris pH 7.5 with boric acid, 2% SDS, 50 mM EDTA, 1% mercaptoethanol). 0.25 volumes of ethanol, 0.11 volumes of 5 M potassium acetate and 1 volume of SEVAG was added to the mixture before it was centrifuged at 18,000 R.P.M. for 30 min at 4°C. The upper aqueous layer was transfered to a new tube and 3 x phenol/chloroform/isoamyl alcohol 50/49/1 extractions were performed on it. To the final upper layer, 2.25 volumes of ethanol was added and the solution was incubated at -20°C for 2 hrs to precipitate all nucleic acids. The solution was subsequently centrifuged at 18,000 rpm for 30 min at 4°C, the pellet washed with 80% ethanol and air dried for 10 min. The pellet was resuspended in 10 mL of DEPC water before the addition of 6 mL of 8 M LiCl. This solution was incubated overnight at -20°C.

The solution was centrifuged at 18,000 rpm for 30 min at 4°C. The RNA pellet was washed with 80% ethanol before being vacuum dried. The pellet was then resuspended in 300 µL of DEPC water and precipitated by adding 2.5 volumes of ethanol, 0.1 volumes of 3 M sodium acetate and incubated at -80°C for 20 min. The solution was centrifuged at 14,000 rpm for 30 min at 4°C, and the pellet was washed twice with 80% ethanol before it was vacuum dried and resuspended in 50 µL of DEPC water.

Amplification of capacc1 and capacc2

Reverse transcription of RNA from ripe papaya fruit tissue was performed using 1 µg total RNA and 2.5 U of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase in a reaction mixture of 10 mM Tris-HCl, pH 8.3 containing 50 mM KCl, 5 mM $MgCl_2$, 1 mM of each dNTP, 1 U RNase inhibitor and 0.75 µM of oligonucleotide primer OLE-4 (Table 1). The reaction mixture was incubated for 30 min at 42°C and then heated to 99°C for 5 min to inactivate the reverse transcriptase. The cDNA produced was amplified with 2.5 U AmpliTaq DNA Polymerase in a reaction mixture of

10 mM Tris-HCl, pH 8.3 containing 2 mM $MgCl_2$, 50 mM KCl, and 0.15 μM of OLE-2 and OLE-4 (Table 1). After an initial 3 min denaturing period at 94°C, the PCR parameters were 1 min template denaturation at 94°C, 1 min primer annealing at 48°C and 2 min primer extension at 72°C for 45 cycles. A final extension step of 15 min at 72°C was used subsequently to ensure full length amplification products.

The products of this PCR reaction were further amplified using a second set of oligonucleotide primers OLE-5 and OLE-6 (Table 1). The reaction consisted of adding to a tube a sample of the products obtained from the previous amplification and 2.5 U AmpliTaq DNA Polymerase in a reaction mixture of 10 mM Tris-HCl, pH 8.3 containing 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dNTP, 0.1% gelatin and 0.15 μM of each primer in a total volume of 100 μL. The PCR parameters were 30 sec template denaturation at 94°C, 30 sec primer annealing at 48°C and 1.5 min primer extension at 72°C for 30 cycles. The PCR products were analyzed on 0.8% agarose gels and visualized with ethidium bromide.

Results:

After reverse transcription and PCR amplification, a DNA band of approximately 1.1 Kb could be observed in electrophoresis gels. The DNA fragment corresponding to this band was purified from the gel and subcloned into pBluescript. Analysis of several recombinant plasmids revealed the presence of two different inserts of approximately the same length but with different restriction patterns. The two different clones were named *capacc1* and *capacc2* respectively. The DNA and deduced amino acid sequences corresponding to *capacc1* and *capacc2* are shown respectively in FIG. 2 and FIG. 3. Comparison of these sequences with other published ACC synthase sequences indicates that *capacc1* and *capacc2* are cDNAs coding for members of the ACC synthase family in papaya. Searches in all the available protein and DNA data banks failed to find 100% homology with any existing clone. The highest homology found for *capacc1* was 74% at the DNA level with a *Pelargonium hortorum* clone (# U17231, GenBank database) and 76% at the protein level with a *Pelargonium hortorum* ACC synthase (# 1124858 GenBank database).

For *capacc2*, the highest homology found at the DNA level was 72% respectively with a *Pelargonium hortorum* mRNA clone (# U17231, GenBank database)

and an *Arabidopsis thaliana* mRNA clone (# M95595, GenBank database). At the protein level, the highest homology found for *capacc2* was 71% with an ACC synthase from *Arabidopsis thaliana* (entry # 1254990, GenBank database).

Analysis of the *capacc1* and *capacc2* DNA sequences reveals that the sizes of *capacc1* and *capacc2* are 1104 bp and 1098 bp respectively. Each of these DNA sequences represents approximately 75% of the coding sequence relating thereto.

EXAMPLE 3

Amplification of ACC Synthase Genes from Mango

MATERIALS AND METHODS

RNA Extraction Procedure for Mango Tissue

Six grams of frozen ripe mango tissue (derived from *Mangifera indica* L.) was ground under liquid nitrogen until it formed a fine powder. The ground tissue was transferred into a falcon tube containing 15 mL extraction buffer (150 mM Tris pH 7.5 with boric acid, 2% SDS, 50 mM EDTA, 1% mercaptoethanol) and vortexed for 2 min. 0.25 vol of ethanol was then added and vortexed for 30 sec. 0.11 vol of 5 M potassium acetate was then added and vortexed for 30 sec. 1 vol SEVAG was then added, vortexed for 1 min and transferred into a DEPC treated centrifuge tube. The tube was centrifuged for 30 min at 18,000 rpm at 4°C. Phenol-SEVAG extractions were performed until no interface layer was observed. The centrifugation steps were performed in falcon tubes for 1 min at 4,500 rpm at 4·C. The supernatant was transferred into a new tube and 2.25 vol of ethanol and the nucleic acids were precipitated at -20°C for 20 min. The tube was centrifuged for 30 min at 18,000 rpm at 4°C in a SS34 rotor. The pellet was washed with 80% ethanol and allowed to dry inverted on a tissue and was subsequently resuspended in 10 mL DEPC water and 6 mL LiCl (8 M) was subsequently added and the RNA was precipitated overnight at -20°C.

The tube was subsequently centrifuged for 30 min at 18,000 rpm at 4°C in a SS34 rotor, the pellet containing the RNA was washed with 80% ethanol and all traces of the ethanol was carefully removed. The pellet was resuspended in 300 µL of DEPC water to which was added 2.5 vol of ethanol and 0.1 vol of 3 M NaAcetate (pH 5.2). The

RNA was precipitated at -80°C for 20 min and the tube was centrifuged in a microfuge at 4°C for 30 min and the pellet washed with 80% ethanol. The pellet was finally resuspended in 50 µL of DEPC water and stored at -80°C.

Amplification of miacc1 and miacc2

Reverse transcription of RNA from ripe mango fruit tissue was performed by using 1 µg total RNA and 2.5 U of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase in a reaction mixture of 10 mM Tris-HCl, pH 8.3 containing 50 mM KCl, 5 mM $MgCl_2$, 1 mM of each dNTP, 1 U RNase inhibitor and 0.75 µM of oligonucleotide primer OLE-4 (Table 1). The reaction mixture was incubated for 30 min at 42°C and then heated to 99°C for 5 min to inactivate the reverse transcriptase. The cDNA produced was amplified with 2.5 U AmpliTaq DNA Polymerase in a reaction mixture of 10 mM Tris-HCl, pH 8.3 containing 2 mM $MgCl_2$, 50 mM KCl, and 0.15 µM of OLE-2 and OLE-4 (Table 1). After an initial 3 min denaturing period at 94°C, the PCR parameters were 1 min template denaturation at 94°C, 1 min primer annealing at 48°C and 2 min primer extension at 72°C for 45 cycles. A final extension step of 15 min at 72°C was used subsequently to ensure full length amplification products.

The products of this PCR reaction were further amplified using a second set of oligonucleotide primers OLE-5 and OLE-6 (Table 1). The reaction consisted of adding to a tube a sample of the products obtained from the previous amplification and 2.5 U AmpliTaq DNA Polymerase in a reaction mixture of 10 mM Tris-HCl, pH 8.3 containing 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dNTP, 0.1% gelatin and 0.15 µM of each primer in a total volume of 100 µL. The PCR parameters were 30 sec template denaturation at 94°C, 30 sec primer annealing at 48°C and 1.5 min primer extension at 72°C for 30 cycles. The PCR products were analysed on 0.8% agarose gels and visualized with ethidium bromide.

Results:

After reverse transcription and PCR amplification, a DNA band of approximately 1.1 Kb could be observed in electrophoresis gels. The DNA fragment corresponding to this band was purified from the gel and subcloned into pBluescript. The analysis of several recombinant plasmids revealed the presence of two different inserts of approximately the same length but with different restriction patterns. The two

different clones were named *miacc1* (1096 bp) and *miacc2* (1113 bp) respectively. The nucleotide and deduced amino acid sequences corresponding to *miacc1* and *miacc2* are shown respectively in FIG. 4 and FIG. 5. Comparison of these sequences with other published ACC synthase sequences indicates that *miacc1* and *miacc2* are cDNAs coding for members of the ACC synthase family in mango. Searches in all the available protein and DNA data banks failed to find 100% homology with any existing clone. The highest homology found for *miacc1* was 71% at the DNA level with the inventor's previously published ACC synthase from mung bean (entry #Z11562 in the EMBL data bank), and 71% at the protein level with a petunia clone # S31450 in the PIR data base. The highest homology found for *miacc2* was 73% at the DNA level respectively with a *Pelargonium hortorum* clone (entry #U17231 in the GenBank database) and a *Vigna radiata* mRNA clone (# Z11562, EMBL data bank). The highest homology found at the protein level for *miacc2* was 77% with an ACC synthase from *Vigna radiata* (entry # S26214, PIR database).

Analysis of the *miacc1* and *miacc2* DNA sequences indicates that each of these sequences represents 75% of the coding region relating thereto.

EXAMPLE 4

Stable Integration and Expression of Acc Synthase Antisense Genes in Papaya (*Carica papaya* L.)

Materials and Methods

Initiation of somatic embryogenesis

Immature zygotic embryos are excised from green fruit of a high yielding papaya line, 90-100 days after pollination and are induced to form somatic embryos. Somatic embryos may be induced by placing zygotic embryos in solution culture consisting of 1/2MS (Murashige and Skoog, 1962, Physiologia Plantarum, **15**, 473-497), 2 μM 6-benzylamino purine (BAP), 0.5 μM naphthaleneacetic acid (NAA), 400 μM adenine sulfate and 3% sucrose, pH 5.65 (Drew *et al.*, 1994, *In* Current Issues in Plant Molecular and Cellular Biology, Proceedings of the VII International Congress on Plant Tissue Culture pp. 321-326, Florence, Italy) on an orbital shaker. Callused cotyledons are removed and discarded after 2-3 weeks. After a further 2 months on the shaker, the media are replaced with EM medium (1/2MS, 0.5 μM BAP, 0.05 μM NAA and 3%

sucrose). Once secondary embryos are formed on this medium, they are multiplied approximately 10 fold on solid EM medium containing 0.8% Difco bactoagar (Drew et al., 1994, *supra*) and maintained on this medium.

Pretreatment of embryos

Somatic embryos are multiplied for three weeks in solid medium and cultured in liquid multiplication medium for three days prior to bombardment. Liquid medium pretreatment of somatic embryos can be performed by culturing embryos for 0-3 days in 30 mL of liquid EM medium at 70 rpm on an orbital shaker.

Transformation vector

A transformation vector which may be utilized for generating a transgenic variety of papaya, wherein expression of an ACC synthase enzyme is substantially inhibited, may be constructed by operably linking the nucleotide sequence of FIG. 2 or FiG. 3, in the antisense orientation, to the CaMV 35S promoter (or other suitable promoter) and to the nopaline synthase terminator region (or other suitable terminator sequence). The vector may also contain the kanamycin resistance gene as selectable marker.

Plasmid DNA for microprojectile bombardment can be purified by two cycles of caesium chloride-ethidium bromide density gradient centrifugation (Sambrook *et al.*, 1989, *supra*).

Bombardment conditions

Embryos are bombarded using a particle inflow gun (Finer *et al.*, 1992, Plant Cell Rep., **11**, 323-328). Gold particles of 1.5-3 μm diameter (Aldrich) are used as microprojectiles wherein 120 mg of such gold particles are washed three times with 100% ethanol and three times with sterile water before suspension in 1 mL of sterile 50% glycerol. For preparation of microprojectiles, 25 μL of the gold suspension is mixed with 0.5 μg plasmid DNA, 25 μL 1 M $CaCl_2$, and 5 μL 0.1 M spermidine free base. All solutions are kept on ice.

The suspension is first sonicated and subsequently kept in suspension by occasional vortexing for 5 minutes. The suspension is then allowed to settle on ice for 10 minutes before 25 μL of the supernatant is moved and discarded. The remaining suspension is subsequently vortexed immediately before utilizing 4 μL of the mixture for each bombardment.

Embryos are arranged, without overlap, in an area of approximately 10 mm diameter. A protective baffle of stainless steel mesh with an aperture of 210 μm (Franks and Birch, 1991, Aus. J. Plant Physiol. **18**, 471-480) is placed over the tissue during bombardment. The pressure of the helium blast is 500 kPa and the distance of the target embryos from the filter unit containing the coated gold particles is 7.5 to 10 cm.

Selection and regeneration of transformed plantlets

Following bombardment, somatic embryos are allowed to recover for 2 days on EM medium, prior to selection on EM medium containing kanamycin monosulfate (200 μg/mL). Embryos are then selected for kanamycin resistance after 3 to 5 months in such medium.

For germination, embryos are transferred to a modified de Fossard's medium (De Fossard *et al.*, 1974, Physioiogia Plantarum, **30**, 125-130), as described by Drew and Miller, (1989, HortScience **64**, 767-773), containing 25 μg/mL kanamycin. Single plantlets are then transferred into individual vessels containing single shoot medium (Drew and Smith, 1986, J. Hort. Sci., **61**, 535-543) without kanamycin.

EXAMPLE 5

Stable Integration and Expression of ACC Synthase Antisense Genes in Mango (*Mangifera indica* L.)

Materials and Methods

Establishment of embryogenic cultures.

Embryogenic cultures are established according to procedures described by Dewald *et al.* (1989, Journal of the American Society of Horticultural Science **114**, 712-716; *ibid*, **114**, 837-841). Immature fruits (4 to 6 cm) of especific mango cultivars (*M. indica L.*) are collected. Fruits are then surface-sterilized with 0.3% (wt/vol) sodium hypochlorite containing 2 to 3 drips of Tween 20 per 100 ml sterilant for 30 min, and subsequently rinsed thoroughly in sterile de-ionized water. Fruits are then bisected longitudinally and zygotic and nucellar embryos are excised and discarded. The ovule halves are then cultured so that the nucelli is in contact with a medium consisting of B5 major salts (Gamborg *et al.*, 1968, Exp. Cell. Res., **50**, 150-158), MS minor salts and organics (Murashige and Skoog, 1962, *supra*), supplemented with 6% (wt/vol) sucrose,

2.7 mM L-glutamine, and 4.5 μM 2.4-dichlorophenoxyacetic acid (2,4-D) (maintenance medium). The pH is then adjusted to 5.8 before addition of 0.17% gelrite (wt/vol) and the medium autoclaved at 120° C at 1.1 $kgcm^{-2}$ for 15 min.

Explants are then transferred to fresh medium after 5 days, 10 days, and at 4-wk intervals thereafter. Proliferating nucellar proembryogenic masses are separated from ovule walls and cultured separately. These may be maintained on solid medium with a subculture interval of 30 days or in liquid medium of the same composition with a subculture interval of 5 days. All cultures on solid medium are kept in darkness at 25°C. Liquid cultures are maintained in the dark for 8 h and 16 h in diffused light (< 0.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 27°C. For *Agrobacterium tumefaciens* infection, actively growing proembryo masses in suspensions are used which have been derived from a 10-month-old culture maintained on solid medium.

Preparation of bacterial culture.

A single colony of *A. tumefaciens*, 9749ASE, from LB agar medium supplemented with 50 μg/ml kanamycin, 25 μg/ml chloramphenicol, and 100 μg/ml spectinomycin is inoculated into 5 mL of liquid broth of the same composition, at pH 7. After 16 h this is transferred to 50 mL of liquid broth of the same composition supplemented with 30 μM acetosyringone, pH 5.6, and incubated at 200 rpm for 16 h at 28° C.

Binary vectors can be constructed by operably linking the nucleotide sequence of FIG. 4 or FIG. 5, in the antisense orientation, to the CaMV 35S promoter (or other suitable promoter) and to the nopaline synthase terminator region (or other suitable terminator sequence) between the left and the right borders. The vector may also contain the NOS-NPTII-NOS chimeric kanamycin resistance gene between the left and the right borders for selection of transformed plant cells.

The recombinant plasmid is introduced into *Agrobacterium* by triparental mating.

Co-cultivation of mango proembryos with A. tumefaciens. Three grams of proembryo masses of size <1000 mm diameter maintained in liquid medium are lightly macerated and cultured in 50 mL of liquid maintenance medium to which 0.05 mL of a log phase culture of acetosyringone-activated *A. tumefaciens* are added. Flasks are maintained on a rotary shaker at 120 rpm. Proembryos are then transferred

to fresh maintenance medium every 24 h for 3 days. No additional bacterium is added at this stage.

Selection protocols.

The main criterium used for identification of transformants is the ability of proembryos to grow in liquid selection medium. The kanamycin levels that are used for the selection are based on earlier studies on kanamycin sensitivity of mango somatic embryos (Mathews and Litz, 1990, HortScience, **25**, 965-966).

After 3 days of co-cultivation, the proembryo masses are selected using a stepwise selection protocol.

Step 1: Proembryos are evenly plated on solid maintenance medium with 200 μg/mL kanamycin and 500 μg/mL cefotaxime (10-12 months).

Step 2: Proembryos are more stringently selected on solid medium at 400 μg/mL kanamycin for increasing the proportion of transformed versus non-transformed cells (2 months).

Step 3: Proembryos are subcultured in liquid maintenance medium with 100 μg/mL kanamycin (2-3 months).

Proembryos on solid medium are then subcultured onto the same medium every 3 wk for 10 months, and thereafter maintained on selection medium without cefotaxine.

Recovery of transformed somatic embryos.

Proembryos from liquid selection medium are transferred to liquid embryogenesis medium (maintenance medium without 2,4-D. supplemented with 0.22 μM benzylaminopurine) containing 100 μg/mL kanamycin for 30 to 50 days for somatic embryo development.

All cultures in liquid selection medium are transferred subsequently at 3- to 5-day intervals to fresh medium, depending on the amount of darkening that has occurred due to oxidation.

The DNA sequences of the invention have utility as targets for the generation of transgenic variants of pineapple, papaya and mango in which the expression of ACC synthase is substantially inhibited to effect suppression of fruit senescence.

Suitable methods for engineering such transgenic plants which have been

described above could be utilized to engineer such transgenic plants. The use of such methods, in concert with the DNA sequences of the invention, will enable the generation of ripening-resistant varieties of pineapple, papaya and mango. It is anticipated that, in comparison to normal varieties of these fruits, the quality, quantity and longevity of the transgenic varieties will be greatly improved for market in both developed and undeveloped countries.

TABLE 1

Degenerate oligonucleotides designed to amplify ACC synthase genes and cDNAs from higher plants.

EZ-2	**SEQ ID NO:11**	5' TA(C/T)TT(C/T)GA(C/T)GG(A/C/G/T)TGGAA(A/G)GC 3'
EZ-4	**SEQ ID NO:12**	5' TC(A/G)TCCAT(A/G)TT(A/C/G/T)GC(A/G)AA(A/G)CA 3'
EZ-5	**SEQ ID NO:13**	5' CA(A/G)ATGGG(A/C/G/T)(C/T)T(A/C/G/T)GC(A/C/G/T)GA(A/G)AA 3'
EZ-6	**SEQ ID NO:14**	5' GC(A/G)AA(A/G)CA(A/C/G/T)AC(A/C/G/T)C(G/T)(A/G)AACCA 3'
EZ-7	**SEQ ID NO:15**	5' AC(A/C/G/T)C(G/T)(A/G)AACCA(A/C/G/T)CC(A/C/G/T)GG(C/T)TC 3'
OLE-2	**SEQ ID NO:16**	5' GCTCTAGATA(C/T)TT(C/T)GA(C/T)GG(A/C/G/T)TGGAA(A/G)GC 3'
OLE-4	**SEQ ID NO:17**	5' GCGAATTC(A/G)TCCAT(A/G)TT(A/C/G/T)GC(A/G)AA(A/G)CA 3'
OLE-5	**SEQ ID NO:18**	5' CCTGATCA(A/G)ATGGG(A/C/G/T)(C/T)T(A/C/G/T)GC(A/C/G/T)GA(A/G)AA 3'
OLE-6	**SEQ ID NO:19**	5' CTCTGCAGC(A/G)AA(A/G)CA(A/C/G/T)AC(A/C/G/T)C(G/T)(A/G)AACCA 3'